Treatment of pigs experimentally infected with *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae* with various antibiotics

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Abstract

The authors have performed a comparative study of the efficacy of various in-feed medications for the treatment of 5- to 6-week-old specific pathogen-free (SPF) piglets experimentally infected on day 1 with *Mycoplasma hyopneumoniae*, on day 8 with *Pasteurella multocida* (serotype A), and on day 15 with *Actinobacillus pleuropneumoniae* (serotype 2). The treatment started on day 9 and continued for 12 consecutive days, then the piglets were euthanized for examination of macroscopic, histologic, and pathologic lesions and for the presence of mycoplasmas and bacteria in the lungs. Based on the results of clinical observations (respiratory signs, rectal temperature, body weight gain, and feed conversion efficiency), macroscopic and histologic lesions of the lungs, and microbiologic findings, the best results were obtained by treatment of pigs with Econor + chlortetracycline, followed by Tetramutin, Pulmotil, Cyfac, and lincomycin + chlortetracycline.

Résumé

Une étude fut entreprise afin de comparer l'efficacité de différentes médicaments administrés dans l'alimentation pour traiter des porcs exempts d'agents pathogènes spécifiques infectés expérimentalement avec Mycoplasma hyopneumoniae *au jour 1, avec* Pasteurella multocida *(sérotype A) au jour 8 et avec* Actinobacillus pleuropneumoniae *(sérotype 2) au jour 15. L'administration de médicaments débuta au jour 9 et se poursuivit pendant 12 jours consécutifs. On procéda alors à l'euthanasie des porcelets et à un examen macroscopique et histopathologique des lésions ainsi qu'à des cultures pour détecter la présence de bactéries et mycoplasmes dans les poumons. Les résultats des observations cliniques (signes respiratoires, température rectale, gain de poids corporel et conversion alimentaire), des examens macroscopiques et histopathologiques des lésions et des cultures bactériennes ont permis de démontrer que les meilleurs résultats furent obtenus suite au traitement des porcs avec Econor + chlortétracycline, suivi par Tetramutin, Pulmotil, Cyfac et lincomycine + tétracycline.*

(Traduit par Docteur Serge Messier)

Introduction

The respiratory disease complex of swine causes major economic losses to swine producers. One of the main etiological factors of this complex is *Mycoplasma hyopneumoniae* (1) which damages the epithelial cells of the respiratory organs (2,3) and inhibits the functioning of the lymphoid system (4,5). *Mycoplasma hyopneumoniae* is considered to play a major role, along with porcine respiratory and reproductive syndrome virus (PRRSV) and swine influenza virus (SIV), in the porcine respiratory disease complex (PRDC) (6), which is now considered to be the most common respiratory disease problem in growing/finishing pigs in the United States (7). Infection by *M. hyopneumoniae* also predisposes the animal to bacterial infection of the respiratory tract.

Among bacterial infections, those by *Pasteurella multocida* (8) and *Actinobacillus pleuropneumoniae* (9) are the most important. The former species has various serotypes (type A or D) which play a role in respiratory disease, while *A. pleuropneumoniae* can be differentiated into 2 Biovars: the B-NAD-dependent Biovar I and the B-NAD-independent Biovar II. Biovar I is more common and contains 12 serotypes based on capsular polysaccharide identification. Distribution of the various serotypes varies from country to country. The most virulent serotypes are 1, 5, 9, 10, and 11, while other serotypes are less virulent and cause a lower level of disease (10).

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Thus, strategically timed, targeted treatment of swine exposed to the PRDC with therapeutic doses of antimicrobials that inhibit both mycoplasmas and bacteria, is a key component of a control program. Combinations of antimicrobials, like tiamulin + chlortetracycline (11), Cyfac (sulfadimidine + chlortetracycline + procaine penicillin), lincomycin + chlortetracycline, and new antibiotics, like tilmicosin and valnemulin (14), have been proposed.

Several authors have published that certain antibiotics or antibiotic combinations are effective against respiratory diseases caused by the aforementioned agents in field conditions. However, very few experimental data are available on testing drugs in a combined *M. hyopneumoniae* + *P. multocida* + *A. pleuropneumoniae* infection model. The purpose of this study was to compare the efficacy of tiamulin + chlortetracycline, valnemulin + chlortetracycline, sulfadimidine + chlortetracycline + procaine penicillin, and lincomycin + chlortetracycline combinations, as well as tilmicosin, for the in-feed treatment of piglets experimentally infected with *M. hyopneumoniae*, *P. multocida*, and *A. pleuropneumoniae*.

Materials and methods

Mycoplasma and bacterial strains used for the experimental challenge

Mycoplasma hyopneumoniae (strain 1230) was isolated in Hungary, using Friis medium (15), from the lungs of a 3-month-old pig showing the characteristic macroscopic and histologic lesions of mycoplasmal pneumonia. The strain was cloned 3 times, identified according to biochemical (positive glucose fermentation, negative arginine hydrolysis, negative phosphatase production) and serological (growth inhibition, metabolic inhibition, epifluorescence, ELISA, applying reference anti-*M. hyopneumoniae* hyperimmune serum) characteristics, and in passage level 6, it was lyophilized and stored at −20°C for 10 y. For the challenge, one ampoule was propagated in Friis medium up to log phase.

Pasteurella multocida strain 185/81, obtained from the lung of a pig suffering from catarrhal pneumonia and preserved in 1:10 glycerol at −20°C, was cultured first on blood agar. Later, the strain was cultured in broth medium and inoculated intranasally (IN) into 2 pigs. The infection caused an increase in temperature and mild sneezing with coughing on day 3 after infection, at which time the animals were euthanized. The strain was recultured for the challenge of the experimental piglets.

Actinobacillus pleuropneumoniae strain 16/96 was cultured from a necrotic focus of 2-month-old pigs having the characteristic macroscopic lesions of *A. pleuropneumoniae* infection. The strain was identified as Biovar I, serotype 2, using biochemical tests and antisera against all the 14 serotypes of Biovar I, as well as those of Biovar II.

Experimental animals

Seventy 5- to 6-week-old PIC piglets were used (*M. hyopneumoniae*-, *A. pleuropneumoniae*-, and toxigenic *P. multocida*-free). The herd of experimental pigs was kept isolated. *Mycoplasma hyopneumoniae*-free status was monitored by: 1) regular serological examination for the presence of *M. hyopneumoniae* antibodies, by a *M. hyopneumoniae* blocking ELISA (DAKO A/S, Glostrup, Denmark), of 50 to 100 sera collected every 6 mo from 4- to 6-month-old pigs; 2) pathomorphologic examination every 2 to 3 mo of the lungs of 50 to 100 slaughterhouse animals for characteristic lesions; and 3) culturing of 10 selected lung samples, on Friis medium, to isolate *M. hyopneumoniae* and by testing these samples by *M. hyopneumoniae*-specific polymerase chain reaction (PCR) (16). Nasal swabs were also collected from undeveloped animals on the farm; these were cultured and tested by PCR. No evidence of *M. hyopneumoniae* infection was found. *Actinobacillus pleuropneumoniae* and *P. multocida* infections were monitored by culturing nasal swabs and lung samples of euthanized animals.

After transportation to the laboratory facilities, the animals were acclimatized to their surroundings for 5 d prior to the initiation of the experiment. The pigs were fed the same feed as was used in the originating farm. Two experiments were performed at different times on experimental piglets of the same origin and the same parameters were examined in both trials.

Experiment 1

The animals were individually marked and body weight was measured. The animals were randomly distributed into 7 groups, 10 animals in each, such that the average body weight of the groups did not differ from each other, according to Student's *t*-test. Six groups of piglets (groups 1 to 6) were infected IN on day 1 of the experiment with an aerosol of 3 mL of *M. hyopneumoniae* broth culture (*M. hyopneumoniae* concentration, 10^7 color-changing units (ccu)/mL). On day 8, animals from these groups were infected IN with an aerosol of 3 mL of Westphal broth culture (2.4×10^9 colony-forming units (cfu)/mL) of *P. multocida* type A, strain 185/91, and on day 15, the animals were infected IN with an aerosol of 3 mL of a trypticase soy broth culture of *A. pleuropneumoniae* strain 16/96 (2.8×10^9 cfu/mL). The infected groups 1, 2, 3, 4, and 5 were medicated via feed (the feed composition was the same for all groups, except that the amount of antibiotic incorporated into the finished feed varied between groups of pigs).

The groups for Experiment 1 were medicated as follows: group 1: valnemulin (Econor, 10% premix, 50 mg/kg feed; Novartis Animal Health GmbH, Kundl, Austria) + chlortetracycline (Aurofac, granular 100, 440 mg/kg feed; Roche Products, Heanor, Derbyshire, United Kingdom); group 2: tiamulin hydrogen fumarate (Tiamutin, 2% premix, 38.5 mg/kg feed; Leo Laboratories, Animal Health Division, Princes Risborough, Bucks, United Kingdom) + chlortetracycline (440 mg/kg feed); group 3: tilmicosin (Pulmotil G200 premix, 400 mg/kg feed; Lilly Industries Limited, Basingstoke, Hampshire, United Kingdom); group 4: chlortetracycline (165 mg/kg feed) + sulfadimidine (165 mg/kg feed) + procaine penicillin (83 mg/kg feed) (Cyfac HS granular; Roche Products); group 5: valnemulin (25 mg/kg feed) + chlortetracycline (440 mg/kg feed); group 6: positive control group (infected, untreated); and group 7: negative control (uninfected, untreated). Treatment started on day 9 and continued for 12 consecutive days. Each group was kept in a separate room. Management, feeding, and water supply were the same for all groups. The animals were slaughtered for postmortem examination on days 22, 23, and 24.

Table I. Clinical scores of pigs experimentally infected with *M. hyopneumoniae*, *P. multocida*, and *A. pleuropneumoniae* and fed with premix formulations of different antibiotics

Experiment 1				Experiment 2			
Group[a]	Total score[b]	Between-group comparison	(*P*-value)[c]	Group[d]	Total score	Between-group comparison	(*P*-value)
1	12	1→2	NS	1	35	1→2	0.01
		1→3	0.01			1→3	NS
		1→4	0.01			1→4	NS
		1→5	NS			1→5	NS
		1→6	0.05			1→6	0.001
		1→7	0.001			1→7	0.001
2	16	2→3	NS	2	89	2→3	0.001
		2→4	0.05			2→4	0.001
		2→5	NS			2→5	0.001
		2→6	0.001			2→6	0.05
		2→7	0.001			2→7	0.001
3	13	3→4	0.01	3	42	3→4	NS
		3→5	NS			3→5	0.05
		3→6	0.001			3→6	0.001
		3→7	0.001			3→7	0.001
4	31	4→5	0.05	4	24	4→5	0.05
		4→6	0.001			4→6	0.001
		4→7	0.001			4→7	0.001
5	15	5→6	0.001	5	114	5→6	0.001
		5→7	0.001			5→7	0.001
6	62	6→7	0.001	6		6→7	0.001
7	0	—	—	7		—	—

[a] Experiment 1: group 1: valnemulin (50 mg/kg) + chlortetracycline (440 mg/kg); group 2: tiamulin (38.5 mg/kg) + chlortetracycline (440 mg/kg); group 3: tilmicosin (400 mg/kg); group 4: sulfadimidine (165 mg/kg) + chlortetracycline (165 mg/kg) + procaine penicillin (83 mg/kg); group 5: valnemulin (25 mg/kg) + chlortetracycline (440 mg/kg); group 6: infected, untreated; group 7: uninfected, untreated

[b] total clinical score for days 8, 15, and 21

[c] comparison made by using the chi-squared test

[d] Experiment 2: group 1: tiamulin (100 mg/kg) + chlortetracycline (400 mg/kg); group 2: lincomycin (100 mg/kg) + chlortetracycline (400 mg/kg); group 3: tilmicosin (300 mg/kg); group 4: valnemulin (25 mg/kg) + chlortetracycline (400 mg/kg); group 5: valnemulin (75 mg/kg) + chlortetracycline (400 mg/kg); group 6: infected, untreated; group 7: uninfected, untreated

Experiment 2

A similar experiment to Experiment 1 was performed. The infected groups 1 to 5 were medicated via feed (the composition of which was the same for all groups except for the type and amount of antibiotic included) as follows: group 1: tiamulin (100 mg/kg feed) + chlortetracycline (400 mg/kg feed); group 2: lincomycin hydrochloride (Lincocin, 100 mg/kg feed; Upjohn SA, Puurs, Belgium) + chlortetracycline (400 mg/kg feed); group 3: tilmicosin (300 mg/kg feed); group 4: valnemulin (25 mg/kg feed) + chlortetracycline (400 mg/kg feed); group 5: valnemulin (75 mg/kg feed) + chlortetracycline (400 mg/kg feed); group 6: positive control (infected, untreated); and group 7: negative control (uninfected, untreated). Each group was kept in a separate room. Management, feeding, and water supply were the same for all groups. The animals were euthanized for postmortem examination on days 22, 23, and 24.

Evaluation of the efficacy of treatment

Clinical observation — Animals were examined on days 8, 15, and 22, at 8 a.m., before feeding or cleaning. Observation was performed for 15 min in each group, examining each individual animal for the presence of clinical signs. Sneezing, coughing, depression, and forced respiration were considered. Clinical signs were scored as 0 (no signs), 1 (mild), and 2 (severe). The total score for each day for one animal was 8. The sums of the scores for the groups on days 8, 15, and 22 were compared statistically (chi-squared test). In the tables, the total scores for each group were compared with the total scores of the other groups; individual animals were not compared.

In each group, rectal temperature was measured every day from days 8 to 17. The average daily value of the temperature was calculated for each group. Those of infected/untreated (group 6) and the infected/treated groups (groups 1 to 5) were compared with those of uninfected/untreated group (group 7) by the Student's *t*-test.

Table II. Occurrence of pneumonia and lung lesion scores of pigs experimentally infected with *M. hyopneumoniae*, *A. pleuropneumoniae*, and *P. multocida* and fed with premix formulations of different antibiotics

	Experiment 1						Experiment 2					
Group[a]	Pneumonia[b]	Between-group comparison (*P*-value)[c]		Lung lesion scores	Between-group comparison (*P*-value)		Pneumonia	Between-group comparison (*P*-value)		Lung lesion score	Between-group comparison (*P*-value)	
1	0/10	1→2	NS	4	1→2	0.01	2/10	1→2	0.05	14	1→2	0.001
		1→3	NS		1→3	0.001		1→3	NS		1→3	NS
		1→4	NS		1→4	0.001		1→4	NS		1→4	NS
		1→5	NS		1→5	NS		1→5	NS		1→5	NS
		1→6	0.01		1→6	0.001		1→6	0.001		1→6	0.001
		1→7	NS		1→7	NS		1→7	NS		1→7	0.001
2	2/10	2→3	NS	14	2→3	NS	6/10	2→3	NS	44	2→3	0.01
		2→4	0.05		2→4	0.001		2→4	NS		2→4	0.001
		2→5	NS		2→5	NS		2→5	NS		2→5	0.001
		2→6	0.001		2→6	0.001		2→6	0.05		2→6	NS
		2→7	NS		2→7	0.001		2→7	0.05		2→7	0.001
3	2/10	3→4	0.05	23	3→4	0.01	5/10	3→4	NS	23	3→4	0.01
		3→5	NS		3→5	0.01		3→5	NS		3→5	0.01
		3→6	0.001		3→6	0.001		3→6	0.01		3→6	0.01
		3→7	NS		3→7	0.001		3→7	0.05		3→7	0.001
4	6/10	4→5	NS	48	4→5	0.001	1/10	4→5	NS	8	4→5	NS
		4→6	NS		4→6	NS		4→6	0.001		4→6	0.001
		4→7	0.05		4→7	0.001		4→7	NS		4→7	0.01
5	1/10	5→6	0.001	6	5→6	0.001	2/10	5→6	0.001	6	5→6	0.001
		5→7	NS		5→7	0.05		5→7	NS		5→7	0.05
6	9/10	6→7	0.001	58	6→7	0.001	10/10	6→7	0.001	58	6→7	0.001
7	0/10	—	—	0	—	—	0/10	—	—	0	—	—

[a] see Table I for breakdown by experiment and by group

[b] number of pigs with pneumonia/number of pigs examined

[c] comparison made by using the chi-squared test

Pathologic examination — The pathologic and histologic examinations were performed such that the examiners were blind as to the treatment group. The lungs of the piglets were examined macroscopically for the presence of lesions characteristic of *M. hyopneumoniae* infection (interstitial pneumonia), complicated with *P. multocida* infection (catarrhal pneumonia), and for the presence of necrotic foci developed due to *A. pleuropneumoniae* infection. Lung lesions were scored from 0 to 5 for each of the apical, cardiac, and diaphragmatic lobes; the maximum score per lung was 30. Since lung lesions associated with *M. hyopneumoniae* infection can be found most frequently in the apical, cardiac, and anterior part of the diaphragmatic lobes, the accessory lobe was not examined. The sums of the scores obtained in the groups were compared by the chi-squared test.

Histologic lesions — Histologic lesions in the right and left apical lobes of the lung were scored as 0 (no lesions), 1 (mild), 2 (medium), and 3 (severe). The first group of lesions included interstitial pneumonia, lymphohistiocytic bronchitis, and peribronchitis; the second was catarrhal bronchopneumonia; and the third was necrotic areas surrounded by connective tissue. The peribronchial lymph nodes were examined for the presence of lymphoid hyperplasia and acute inflammation and the nasal mucosae were examined for lymphohistiocytic infiltration and catarrhal (purulent) inflammation. The maximum lung score per animal was 3 (types of lesions) × 3 (scores) × 2 (lobes) = 18.

Immunofluorescence examination of lungs for M. hyopneumoniae — Sections of lung, from each of the apical, cardiac, and diaphragmatic lobes, were examined for the presence of *M. hyopneumoniae* by immunofluorescence (IF) by using fluorescein isothiocyanate-labeled anti-*M. hyopneumoniae* rabbit serum (17). The intensity of the immunofluorescence was scored as 0 (no fluorescence), 1 (mild), 2 (medium), or 3 (intense) in each of the lobes. The maximum score per lung was 18. The number of IF-positive lobes and IF scores for the groups were compared by the chi-squared test.

Bacteriologic examination — Nasal swabs were taken from the animals before *M. hyopneumoniae* infection on day 1, before *P. multocida* challenge on day 8, and before *A. pleuropneumoniae* infection on day 15. Swabs were examined for the presence of *M. hyopneumoniae* using the technique described by Friis (15), with subsequent identification by an immunofluorescence technique (18). The swabs were examined for *P. multocida* by applying blood agar containing 5% cattle erythrocytes and 0.5% yeast extract, and for *A. pleuropneumoniae* by using trypticase soy agar containing

Table III. Histological lesions in the lungs of piglets experimentally infected with *M. hyopneumoniae*, *A. pleuropneumoniae*, and *P. multocida* and fed with premix formulations of different antibiotics

Type of lesion	Experiment	Group 1	2	3	4	5	6	7
Lymphohistiocytic bronchitis and peribronchitis + interstitial pneumonia	1	14	16	6	6	12	28	0
	2	16	33	29	6	7	63	0
Catarrhal pneumonia	1	0	2	2	6	1	36	0
	2	7	39	39	5	4	20	0

yeast extract (19). At the end of the experiment, lung samples from each animal were also examined for the presence of the microorganisms. The reisolated bacteria were identified according to their biochemical characteristics (catalase, hyaluronidase, oxidase, urease, and indole activities; acid production from glucose, lactose, mannitol, rafinose, salicin, trachanose, and xylose; production of hydrogen sulphide; reduction of nitrate; and the requirement for X and V factors). The serotype of the isolates were identified by passive hemagglutination by using hyperimmune sera produced against reference strains of each species. The total reisolation rate of the mycoplasmas and the bacteria were compared by the chi-squared test.

Serological examination of M. hyopneumoniae *antibodies* — Blood samples were taken from each pig before *M. hyopneumoniae* challenge on day 1, as well as 3 wk after challenge. Antibodies against *M. hyopneumoniae* were detected by a blocking ELISA using anti-P72 kDa monoclonal antibodies (DAKO) (20).

Body weight gain — The experimental piglets were weighed on day 1 before *M. hyopneumoniae* challenge and before slaughter at the end of the experiment. The average body weight gains of the groups during the experiment were compared by the Student's *t*-test.

Feed conversion efficiency — The feed consumption of the animals in each group was measured during the experiment. The feed conversion efficiency (FCE) of each group was calculated as follows:

$$\text{FCE} = \frac{\text{feed consumed (kg)}}{\text{group weight gain (kg)}}$$

Results

Clinical examination

Before infection, no clinical signs were observed; however, on day 8 in all infected groups, but not in the uninfected control group, some animals showed mild signs of respiratory disease. In Experiment 1, on days 15 and 22, more animals showed clinical signs, such as sneezing, depression, forced breathing, and coughing noted in group 6 (positive control group). Among the treated groups, the highest clinical scores were observed in group 4, while pigs in groups 1, 2, 3, and 5 were significantly less affected. A statistically significant difference was observed between groups 4 and 6 (Table I).

In Experiment 2, high clinical scores were again noted in group 6. The next highest scores were noted in the group treated with lincomycin + chlortetracycline (group 2). The scores recorded for groups 1, 3, 4, and 5 were much lower (Table I).

Temperatures

In the non-infected control (group 7) of Experiment 1, the daily average temperatures varied between 38.38 ± 0.2°C and 38.93 ± 0.2°C during the observation period. No significant increase of average temperature was noticed in groups 1 and 5 when compared with group 7. By contrast, in groups 4 and 6, a significant increase in temperature was observed in comparison with the value measured in group 7; values of 39.37 ± 0.1°C and 39.73 ± 0.2°C, respectively, were obtained on day 3 after *P. multocida* infection, and values of 39.46 ± 0.2°C, 39.70 ± 0.3°C, 39.50 ± 0.2°C and 39.50 ± 0.2°C, 40.23 ± 0.4°C, 39.59 ± 0.2°C, respectively, were obtained on days 2, 3, and 4 after *A. pleuropneumoniae* infection. In group 3, a significant temperature increase was noticed only on day 3 after *P. multocida* infection and on day 1 after *A. pleuropneumoniae* infection (39.56 ± 0.2°C and 39.47 ± 0.1°C).

In Experiment 2, no increase of temperature was noticed in groups 1, 4, and 5. By contrast, in group 6 (positive control) during the first 3 d after *P. multocida* infection, 3 d after *A. pleuropneumoniae* infection, significant increase of temperature (39.23 ± 0.1°C, 39.9 ± -0.3°C, 39.37 ± 0.2°C and 39.50 ± 0.2°C, 40.23 ± 0.4°C, 39.59 ± 0.3°C, respectively) was observed in comparison with the values measured in group 7, where daily average values varied between 38.38 ± 0.3°C and 39.71 ± 0.4°C. On the other hand, in group 2, the average rectal temperatures were significantly higher than the negative control group 1 d after *P. multocida* (39.74 ± 3.1°C) and 2 d after *A. pleuropneumoniae* infection (39.46 ± 0.5°C and 39.70 ± 0.5°C), respectively. For group 3, the average rectal temperatures were significantly higher than the negative control group 1 d after *P. multocida* (39.3 ± 0.2°C) and 1 d after *A. pleuropneumoniae* challenge (39.54 ± 0.3°C).

Pathologic lesions

The number of animals with catarrhal pneumonia, bronchitis, and necrotic foci, as well as their lesion scores, are presented in Table II. In Experiment 1, all animals in group 6 showed macroscopic pathologic lesions. By contrast, a significantly lower number of animals with gross lesions was seen in the treated groups, with the exception of group 2. A similar picture was seen with regard to macroscopic lesion scores. The lowest lesion scores were noticed in groups 1 and 5, better than those for groups 2 and 3.

Table IV. Results of *M. hyopneumoniae* isolation from nasal swabs 2 and 3 wk after infection and scores of immunofluorescence examination of lungs of piglets experimentally infected with *M. hyopneumoniae*, *P. multocida*, *A. pleuropneumoniae* and fed with premix formulations of different antibiotics

	Experiment 1						Experiment 2					
Group[a]	Isolation of M.h.[b]	Between-group comparison (*P*-value)[c]		Sum of IF scores	Between-group comparison (*P*-value)		Isolation of M.h.	Between-group comparison (*P*-value)		Sum of IF scores	Between-group comparison (*P*-value)	
1	0/20	1→2	0.01	10	1→2	0.001	0/20	1→2	0.01	47	1→2	0.001
		1→3	0.01		1→3	0.001		1→3	0.01		1→3	0.01
		1→4	0.001		1→4	0.001		1→4	NS		1→4	0.001
		1→5	NS		1→5	NS		1→5	NS		1→5	0.001
		1→6	0.001		1→6	0.001		1→6	0.001		1→6	0.001
		1→7	NS		1→7	0.01		1→7	NS		1→7	0.001
2	4/20	2→3	NS	30	2→3	NS	6/20	2→3	NS	105	2→3	0.01
		2→4	0.05		2→4	0.001		2→4	0.05		2→4	0.001
		2→5	NS		2→5	0.001		2→5	0.05		2→5	0.001
		2→6	0.01		2→6	0.001		2→6	NS		2→6	0.001
		2→7	NS		2→7	0.001		2→7	0.05		2→7	0.001
3	4/20	3→4	0.01	44	3→4	0.001	4/20	3→4	NS	75	3→4	0.001
		3→5	NS		3→5	0.001		3→5	NS		3→5	0.001
		3→6	0.01		3→6	0.001		3→6	NS		3→6	0.001
		3→7	NS		3→7	0.001		3→7	NS		3→7	0.001
4	11/20	4→5	0.01	102	4→5	0.001	0/20	4→5	NS	15	4→5	NS
		4→6	NS		4→6	0.001		4→6	0.01		4→6	0.001
		4→7	0.001		4→7	0.001		4→7	NS		4→7	0.001
5	1/20	5→6	0.001	9	5→6	0.001	4/20	5→6	0.01	9	5→6	0.001
		5→7	NS		5→7	0.01		5→7	NS		5→7	0.01
6	12/20	6→7	0.001	151	6→7	0.001	8/20	6→7	0.01	152	6→7	0.001
7	0/20	—	—	0	—	—	0/20	—	—	0	—	—

M.h. — *M. hyopneumoniae*; IF — immunofluorescence

[a] see Table I for breakdown by experiment and by group

[b] number of pigs with *M. hyopneumoniae* isolated/number of pigs examined

[c] comparison made by using the chi-squared test

Similarly in Experiment 2, all animals in group 6 showed macroscopic pathologic lesions. By contrast, groups 2 and 3 had a significantly lower number of animals with gross lesions, but the lowest numbers were noticed in groups 1, 4, and 5. A similar picture was seen in relation to macroscopic lesion scores, a significant difference being detected between groups 2 and 3 and the positive control group. The lesion scores of groups 1, 4, and 5 were similar, but significantly lower in comparison with those of group 3.

Histologic lesions

Interstitial pneumonia, lymphohistiocytic bronchitis and the formation of lymphoid follicles are associated with *M. hyopneumoniae* challenge; necrotic foci, proliferation of macrophages and fibrosis are due to *A. pleuropneumoniae* while catarrhal pneumonia and bronchitis are attributed to *P. multocida* infection. In Experiment 1, all medicated animals had slight lesions characteristic of interstitial pneumonia, lymphohistiocytic bronchitis and peribronchitis (Table III). The scores of these lesions did not differ from those of the control, uninfected group 7. It should be emphasised that these lesions were highly pronounced in Experiment 2, and consequently the lesion score was significantly higher, in the infected untreated group 6. Similarly, high scores of catarrhal pneumonia and necrotic areas were also seen in this group, whilst no such lesions were observed in the medicated groups. Hyperplasia of the peribronchial lymph nodes as well as lymphohistiocytic infiltration of the nasal mucosa were also seen in all animals of groups 3 and 6. Acute inflammation of lymph nodes was observed only in group 6.

Immunofluorescence examination of lungs for *M. hyopneumoniae*

In Experiment 1, a significant reduction of the number of IF-positive lobes and IF scores was observed in all treated groups in comparison with control group 6. The lowest number of positive lobes and IF scores were found in groups 1 and 5, followed by groups 2, 3, and 4 (Table IV). In Experiment 2, the number of IF-positive lobes was similar in group 2 and control (group 6), while a significant reduction of the number of IF-positive lobes was observed in the other infected treated groups. The smallest IF score was found in group 5 followed by group 4, group 1, and group 3.

Table V. Results of *M. hyopneumoniae* and *A. pleuropneumoniae* re-isolation from the lungs of piglets experimentally infected with *M. hyopneumoniae*, *P. multocida*, *A. pleuropneumoniae* and fed with premix formulations of different antibiotics

	Experiment 1						Experiment 2					
Group[a]	Isolation of M.h.[b]	Between-group comparison (*P*-value)[c]		Isolation of A.pp.[b]	Between-group comparison (*P*-value)		Isolation of M.h.	Between-group comparison (*P*-value)		Isolation of A.pp.	Between-group comparison (*P*-value)	
1	0/10	1→2	0.01	0/10	1→2	NS	0/10	1→2	0.01	1/10	1→2	NS
		1→3	0.05		1→3	NS		1→3	0.05		1→3	NS
		1→4	0.001		1→4	NS		1→4	NS		1→4	NS
		1→5	NS		1→5	NS		1→5	NS		1→5	NS
		1→6	0.001		1→6	0.001		1→6	0.001		1→6	0.01
		1→7	NS		1→7	NS		1→7	NS		1→7	NS
2	4/10	2→3	NS	0/10	2→3	NS	5/10	2→3	NS	2/10	2→3	NS
		2→4	0.01		2→4	NS		2→4	0.05		2→4	NS
		2→5	NS		2→5	NS		2→5	0.05		2→5	NS
		2→6	0.01		2→6	0.001		2→6	NS		2→6	0.05
		2→7	NS		2→7	NS		2→7	0.05		2→7	NS
3	2/10	3→4	0.001	0/10	3→4	NS	3/10	3→4	NS	1/10	3→4	NS
		3→5	NS		3→5	NS		3→5	NS		3→5	NS
		3→6	0.001		3→6	0.001		3→6	0.01		3→6	0.01
		3→7	NS		3→7	NS		3→7	NS		3→7	NS
4	9/10	4→5	0.001	2/10	4→5	NS	0/10	4→5	NS	0/10	4→5	NS
		4→6	NS		4→6	0.01		4→6	0.001		4→6	0.01
		4→7	0.001		4→7	NS		4→7	NS		4→7	NS
5	1/10	5→6	0.001	0/10	5→6	0.001	0/10	5→6	0.001	1/10	5→6	0.01
		5→7	NS		5→7	NS		5→7	NS		5→7	NS
6	10/10	6→7	0.001	7/9	6→7	0.001	8/10	6→7	0.01	6/10	6→7	0.001
7	0/10	—	—	0/10	—	—	0/10	—	—	0/10	—	—

M.h. — *M. hyopneumoniae*; A.pp. — *A. pleuropneumoniae*

[a] see Table I for breakdown by experiment and by group

[b] number of pigs with *M. hyopneumoniae* (or *A. pleuropneumoniae*) isolated/number of pigs examined

[c] comparison made by using the chi-squared test

Bacteriologic examination

The results of the reisolation of *M. hyopneumoniae* from nasal swabs from the experimental piglets are described in Table IV. Before *M. hyopneumoniae* challenge, all animals were culture negative. Taking into consideration the total isolation rate 2 and 3 wk after challenge, there was no significant difference in the number of *M. hyopneumoniae* from nasal swabs observed in group 4 when compared with that of control group 6, while a significant difference was shown in groups 1, 2, 3, and 5. The lowest isolation rates were obtained in groups 1 and 5, a higher isolation rate was noted in groups 2 and 3.

In Experiment 2 (12 d after starting the treatment), taking into consideration the total isolation rate 2 and 3 wk after challenge, mycoplasmas could not be reisolated from groups 1 and 4. No significant reduction of *M. hyopneumoniae* from nasal swabs was observed in groups 2 and 3, in comparison with those from control group 6.

In Experiment 1, *M. hyopneumoniae* could not be cultured from the lungs of piglets belonging to group 1 (Table V). A significant reduction was seen in the re-isolation rate of *M. hyopneumoniae* from the lungs of groups 2, 3, and 5 in comparison with the control group 6, while the isolation rate in group 4 and control group 6 were not statistically different from each other. *Actinobacillus pleuropneumoniae* was successfully isolated, in a relatively high proportion, from the lungs of piglets of group 6, while only 2 animals proved to be positive in group 4 (Table V).

In Experiment 2, mycoplasmas could not be detected in the lung samples from groups 1, 4, and 5. A significant reduction however, was noticed in group 3, but not in group 2. In both experiments, *A. pleuropneumoniae* reisolation was significantly lower in all the treated groups than that noted in the infected, untreated group 6.

Serological response of piglets to *M. hyopneumoniae*

All serum samples taken before *M. hyopneumoniae* challenge proved to be serologically negative for *M. hyopneumoniae* antibodies. In Experiment 1, among sera collected 3 wk after infection, antibodies were detected in 10 and 8 animals in groups 6 (control) and 4, respectively, whereas none of the animals in groups 1, 2, 3, or 5 were found to be positive.

In Experiment 2, antibodies were detected in 9 of 10 animals in control group 6 and in only 4 of 10 pigs in group 2. No antibodies could be detected in animals in groups 1, 3, 4, or 5.

Table VI. Body weight gains and FCE during experimental period of pigs experimentally infected with *M. hyopneumoniae*, *P. multocida*, and *A. pleuropneumoniae* and fed with premix formulations of different antibiotics

	Experiment 1				Experiment 2			
Group[a]	Body weight gain (kg)	Between-group comparison (*P*-value)[b]		FCE	Body weight gain (kg)	Between-group comparison (*P*-value)		FCE
1	6.24 ± 1.6	1→2	NS	2.05	10.1 ± 2.4	1→2	NS	1.90
		1→3	NS			1→3	NS	
		1→4	NS			1→4	NS	
		1→5	NS			1→5	NS	
		1→6	0.001			1→6	NS	
		1→7	NS			1→7	NS	
2	5.94 ± 1.9	2→3	NS	2.28	8.65 ± 2.93	2→3	NS	2.23
		2→4	NS			2→4	0.05	
		2→5	NS			2→5	NS	
		2→6	0.01			2→6	NS	
		2→7	NS			2→7	NS	
3	5.63 ± 0.6	3→4	NS	2.24	9.95 ± 1.8	3→4	NS	1.88
		3→5	NS			3→5	NS	
		3→6	0.001			3→6	NS	
		3→7	NS			3→7	NS	
4	4.05 ± 2.0	4→5	NS	2.48	10.94 ± 1.47	4→5	NS	1.87
		4→6	NS			4→6	0.001	
		4→7	NS			4→7	NS	
5	5.73 ± 1.1	5→6	0.001	2.19	10.56 ± 2.72	5→6	NS	1.83
		5→7	NS			5→7	NS	
6	4.05 ± 1.4	6→7		2.83	8.85 ± 1.6	6→7	NS	2.44
7	5.83 ± 1.0	—	—	2.22	10.00 ±	—	—	1.93

FCE — feed conversion efficiency

[a] see Table I for breakdown by experiment and by group

[b] comparison made by using the Student's *t*-test

Body weight gain

In Experiment 1, the body weights of the groups were not statistically different from each other prior to the study. The body weight gains of group 6 were the lowest (Table VI). The highest body weight gains were observed in groups 1, 5, and 3. No statistically significant differences were observed between them, but the weight gains for these 3 groups were significantly higher than that of the control group 6. Only group 4, treated with Cyfac, did not have a statistically significant increase in weight gain when compared with control group 6.

In Experiment 2, the body weight gains of animals in groups 1, 3, 4, and 5 were similar. Lower body weight gains were recorded in group 2 and did not differ statistically from those of control group 6.

Feed conversion efficiency

Feed conversion efficiency values were calculated for the experimental period and are shown in Table VI. In Experiment 1, during treatment, the FCE of control group 6 increased to 2.83. On the other hand, the FCE of the treated groups was lower, particularly in groups 1 and 5, being lower than that of the negative control group 7. The FCE in group 4 was high.

In Experiment 2, the FCE of the positive control group 6 increased the most (2.44), followed by group 2 (2.23). On the other hand, the FCE of groups 1, 3, 4, and 6 proved to be at a similar level and were lower than those of control group 7.

Discussion

In many countries, respiratory disease in grow-finish pigs is frequently referred to as the porcine respiratory disease complex (PRDC), a complex involving viruses, mycoplasmas, and bacteria. The primary pathogens involved are PRRSV, SIV, pseudorabies virus (PRV), porcine circovirus (PCV), *M. hyopneumoniae*, *A. pleuropneumoniae*, *Bordetella bronchiseptica*, and *P. multocida*. Opportunistic mycoplasmal and bacterial pathogens include: *Streptococcus suis*, *Haemophilus parasuis*, *Mycoplasma hyorhinis*, and most strains of *P. multocida* (21). Recent research (22) has shown that *M. hyopneumoniae* has a pivotal role in the pathogenesis of porcine respiratory disease (PRD). It was demonstrated experimentally that *M. hyopneumoniae* potentiates the severity and duration of PRRSV-induced pneumonia. Further, it has been shown that vaccination with a modified live virus vaccine, or infection with PRRSV, during or following vaccination with a *M. hyopneumoniae* vaccine, appears to reduce the efficacy of the latter vaccine (23). Although current vaccines for *M. hyopneumoniae* decrease clinical disease, it is thought that new vaccines that decrease colonization of *M. hyopneumoniae* are needed. Therefore, in attempts to control PRDC, antibiotics that

inhibit the major mycoplasmal and bacterial components, in addition to vaccines and manipulations of management, clearly have an important role.

In this model infection involving *M. hyopneumoniae*, *P. multocida* and *A. pleuropneumoniae*, a group of in-feed antibiotics were compared for their efficacy, judged by clinical, microbiologic, pathologic, and productivity criteria. It was of interest, in view of the significance now attached to the role of *M. hyopneumoniae* in the development of PRD, that the groups in the study medicated with tiamulin and valnemulin + chlortetracycline showed a major reduction in mycoplasma contamination levels in the respiratory tract when compared with the unmedicated controls and other medicated groups. Since PRRSV is now widespread, it might be useful to conduct similar studies by using a mixed virus, mycoplasma, and bacteria model.

In conclusion, in the study presented, we attempted to reproduce a mycoplasmal and bacterial respiratory disease complex by experimental infection of piglets with *M. hyopneumoniae*, *P. multocida* and *A. pleuropneumoniae*. Taking into consideration the very high virulence of some serotypes of *A. pleuropneumoniae* Biovar I, we selected the less pathogenic serotype 2. This serotype is very common but does not produce high mortality, though it did induce catarrhal-purulent foci in the lungs of the experimental piglets. Due to the induced infection by *M. hyopneumoniae*, *P. multocida* and *A. pleuropneumoniae*, animals in the positive control group exhibited mild sneezing, coughing, depression, and increased body temperature. At necropsy, most of them showed macroscopic lesions, such as purulent foci in the diaphragmatic lobes of the lung (and sometimes in the cardiac lobes), as well as catarrhal pneumonia of varying extent in the cardiac and apical lobes of the lung, with the presence of *M. hyopneumoniae*, *P. multocida*, and *A. pleuropneumoniae*.

Feed medication with the sulfadimidine + chlortetracycline + procaine penicillin combination produced some reduction in the severity of the above-mentioned clinical signs, as did medication with lincomycin + chlortetracycline; however, a more pronounced efficacy was observed with tilmicosin medication and a better one still with tiamulin + chlortetracycline or valnemulin + chlortetracycline. The tiamulin + chlortetracycline results in relation to *A. pleuropneumoniae* infection are in accordance with observations reported by Morgan et al (24), showing the clinical effectiveness of Tetramutin (tiamulin 100 ppm + chlortetracycline 300 ppm) in the prevention of experimentally induced *A. pleuropneumoniae* infection. This also confirmed earlier field observations (11). They reported in vivo and in vitro synergism between tiamulin and chlortetracycline against *A. pleuropneumoniae*. These data are also in agreement with data published by Barfod et al (25), demonstrating the efficacy of tiamulin in controlling respiratory disease induced by *A. pleuropneumoniae*.

Nevertheless, though tiamulin alone has a relatively high minimum inhibitory concentration (MIC) value against *A. pleuropneumoniae* strains (26), a combination of tiamulin + chlortetracycline has a synergistic effect in vitro, as was demonstrated by earlier studies. The satisfactory clinical efficacy of tilmicosin recorded against *A. pleuropneumoniae* is also in accordance with both in vitro testing, showing a low MIC value for this antibiotic against *A. pleuropneumoniae* (27,28), as well as a good distribution of the antibiotic to the site of infection via the macrophage cell system (29).

The clinical efficacy of tilmicosin against *M. hyopneumoniae*, however, was shown in this study to be inferior to the efficacy of valnemulin + chlortetracycline against *M. hyopneumoniae*. The MIC of tilmicosin against *M. hyopneumoniae* is reported to be 1.6–2.0 μg/mL (27,28), whereas valnemulin has an exceptionally low MIC range of 0.00025–0.0005 μg/mL (14). Chlortetracycline alone, at an in-feed inclusion level of approximately 550 mg/kg feed, administered for 14 consecutive days, starting 3 d prior challenge with *M. hyopneumoniae*, has been reported to reduce the occurrence of pneumonia induced by *M. hyopneumoniae* by 95% compared with non-medicated controls. In addition, in-feed administration of chlortetracycline reduced the number of *M. hyopneumoniae* organisms in the lungs (30). Pronounced in vitro synergism between valnemulin and chlortetracycline against *M. hyopneumoniae* has been reported (31). The excellent results noted in the valnemulin-treated groups, at 25 mg/kg feed, 50 mg/kg feed, and 75 mg/kg feed, in combination with chlortetracycline, are probably due to the extremely low MIC against *M. hyopneumoniae*, the synergistic effect of the 2 antibiotics, and the favorable distribution of valnemulin to the target tissue following oral administration, resulting in high efficacy at the site of infection.

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